Supporting Information

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A Selective Chemical Probe for Coenzyme-A Requiring Enzymes

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General
All commercially available reagents were used as purchased without further purification. Unless otherwise noted, all the chemical reagents were purchased from Fisher (Fairlawn, NJ) and Sigma-Aldrich (St. Louis, MO). Hyperfilm ECL was purchased from Amersham Biosciences (Piscataway, NJ). Supersignal West Pico chemiluminescence reagent and horseradish peroxidase (HRP)-conjugated streptavidin were from Pierce (Rockford, IL) Dulbecco’s modified Eagle media (DMEM), fetal bovine serum and penicillin/streptomycin were from Gibco (Carlsland, CA). CoA, 3’-dephospho CoA, desthiobiotin were from Sigma-Aldrich (St. Louis, MO). Nitrocellulose membrane was from Bio-Rad Laboratories, Hercules, CA). Anti-Hat1 and anti-goat IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Sequencing grade trypsin was from Roche Diagnostics (Penzberg, Germany). The bifunctional human PPAT/DPCK enzyme was prepared as a recombinant protein as described previously.[1] Recombinant Csk, Src (aa 81-527, K295M), LMW-PTP, 14-3-3, AANAT, PCAF HAT domain (aa 493-676), GCN5 HAT domain (aa 99-262), p300 HAT domain (aa 1284-1673) and semi-synthetic p300-HAT domain were produced as described previously.[2-9] yESAI HAT domain (aa 160-435) was kindly provided by Dr Ronen Marmorstein. Nuclear magnetic resonance spectra were obtained with a Varian Mercury 400 MHz instrument. HRMS were obtained at the UC Riverside MS facility.

1. Synthesis of Fact-CoA and biotin-CoA-SO probes

Scheme S1. Synthesis of 32P-Fluoroacetonyl-CoA

3’-dephospho-CoASH $\xrightarrow{32P-ATP}$ 3'-32P-CoASH $\xrightarrow{DPC}$ 3'-32P-CoASH $\xrightarrow{F}$ 3'-32P-CoASH

32P-Fluoroacetonyl CoA (32P-FactCoA, I) Fluorochloroacetone[11] (20 µl, 1 M) and 3'-32P-CoASH (200 µl, 10 mM, prepared from 3’- dephosphoCoASH and 32P-ATP by reacting with recombinant DPCK enzyme[11]) were mixed and incubated for 2 h at rt). The mixture was then precipitated with diethyl ether and pelleted by centrifugation. The precipitate was dissolved in ddH2O (400 µl) to make 5 mM aqueous solution for the assay.

For non-radioactive compound: MALDI-HRMS (M+H+) calcd for C24H39N7O17P 842.1431, found m/z 842.1393.

1H NMR (400MHz, D2O) d 8.43 (s, 1H), 8.17 (s, 1H), 5.97 (d, J=5.2Hz, 1H), 4.97 (d, J=46.8Hz, 2H), 4.61 (m, 2H), 4.55 (m, 2H), 4.37 (m, 1H), 4.02 (m, 2H), 3.79 (s, 1H), 3.62 (dd, J=4.8Hz, 9.6Hz, 2H), 3.35 (dd, J=4.8Hz, 9.6Hz, 1H), 3.29 (2H), 3.10 (m, 2H), 2.41 (t, J=6.4Hz, 2H), 2.23 (t, J=6.4Hz, 2H), 0.67 (s, 3H), 0.57 (s, 3H); 31P NMR (160 MHz) d 1.65 (s), -9.71 (d, J=21.0Hz), -10.27 (d, J=21.0Hz).

[3-(Ethylsulfanylcarbonyl-methyl-amino)-propyl]-carbamic acid tert-butyl ester, 4 A solution of ethyl chlorothioformate (1.0 g, 8.0 mmol) in diethyl ether (4 ml) was added dropwise to (3-methylamino-propyl)-carbamic acid tert-butyl ester[12] (3, 1.51 g, 8.0 mmol) in a mixture of diethyl ether (20 ml) and aqueous NaOH (8 ml, 1 M). After 30 min, addition of water (20 ml), followed by phase
separation and washing the organic phase with ddH$_2$O, dilute hydrochloric acid (0.1 M), and ddH$_2$O sequentially, drying over anhydrous Na$_2$SO$_4$ and concentration lead to a clear liquid (2.18 g, 99% yield). ES-TOF HRMS (M+Na$^+$) calcd for C$_{12}$H$_{24}$N$_2$O$_3$NaS 299.1405, found m/z 299.1401.

1H NMR (400MHz, CDC13) d 5.30 (s, 1H), 3.48 (m, 2H), 3.09 (m, 2H), 2.96 (s, 3H), 2.92 (q, J=7.6Hz, 2H), 1.69 (m, 2H), 1.44 (s, 9H), 1.29 (t, J=7.6Hz, 3H); $^{13}$C NMR (100MHz, CDCl3) d 169.17, 156.08, 79.00, 46.16, 37.12, 34.63, 28.43, 27.46, 24.79, 15.41.

Desthiobiotin thiocarbamate

Desthiobiotin (214 mg, 1.0 mmol) was dissolved in a 1:1:1 mixture of DMF/dioxane/ddH$_2$O (6 ml). To the solution, diisopropylethylamine (523 µl, 3.0 mmol) and N,N,N',N'-tetramethyl-O-(Nsuccinimidyl)uranium tetrafluoroborate (TSTU, 380 mg, 1.26 mmol) were added. The mixture was stirred for 3.5 h at rt. Separately, the compound above was subjected to Boc hydrolysis in aqueous methanolic HCl (6 N aqueous HCl : methanol=1/5 (v/v)) for 3 h at rt. After removing the solvent under reduced pressure, the concentrate dissolved in DMF (1.5 ml) and diisopropylethylamine (300 µl) was added to the activated desthiobiotin mixture and stirred for additional 2 h at rt. The reaction mixture was concentrated, directly loaded onto a silica gel column and eluted with methanol and methylene chloride (1:20 v/v) to give a foamy material (250 mg, 67% yield).

ES-TOF HRMS (M+H$^+$) calcd for C$_{17}$H$_{33}$N$_4$O$_3$S 373.2273, found m/z 373.2267.

$^1$H NMR (400MHz, CDCl$_3$) d 6.82 (s, 1H), 6.04 (s, 1H), 5.49 (s, 1H), 3.84 (m, 1H), 3.69 (m, 1H), 3.48 (m, 2H), 3.20 (m, 2H), 2.67 (s, 3H), 2.53 (q, J=7.6Hz, 2H), 2.21 (t, J=7.6Hz, 2H), 1.66 (m, 4H), 1.32-1.50 (m, 6H), 1.29 (t, J=7.6Hz, 3H), 1.12 (d, J=6.8Hz, 3H); $^{13}$C NMR (100MHz, CDCl$_3$) d 173.31, 172.97, 164.22, 56.05, 51.42, 46.17, 36.27, 35.67, 34.67, 29.52, 28.81, 26.87, 25.91, 25.38, 24.81, 15.68, 15.39.

Desthiobiotin sulfone, 5

A solution of Oxone® (369 mg) in ddH$_2$O (3 ml) was added to a cooled solution of desthiobiotin thiocarbamate (110 mg, 0.3 mmol) in methanol (3 ml). The resulting cloudy slurry was stirred for 4 h at rt. The reaction mixture was diluted with ddH$_2$O (40 ml) and extracted 3 times with chloroform (20 ml). The combined extracts were washed with ddH$_2$O and brine, dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure to give a clear oil (64.3 mg, 83% yield).

ES-TOF HRMS (M+H$^+$) calcd for C$_{17}$H$_{33}$N$_4$O$_5$S 405.2172, found m/z 405.2160.

$^1$H NMR (400MHz, CDCl$_3$) d 6.75 (t, J=5.6Hz, 1H), 6.65 (t, J=6.0Hz, 1H), 6.08 (s, 1H), 5.94 (s, 1H), 5.34 (s, 1H), 5.32 (s, 1H), 3.84 (m, 2H), 3.77 (t, J=7.2Hz, 2H), 3.69 (m, 2H), 3.46 (t, J=7.2Hz, 2H), 3.38 (q, J= 7.2Hz, 4H), 3.35 (s, 3H), 3.26 (m, 4H), 3.02 (s, 3H), 2.19 (m, 4H), 1.91 (m, 2H), 1.82 (m, 2H), 1.65 (m, 4H), 1.43 (t, J=7.2Hz, 6H), 1.26-1.51 (m, 12H), 1.12 (d, J=6.8Hz, 3H); $^{13}$C NMR (CDCl$_3$, 100MHz) d 173.53, 173.39, 164.16, 164.10, 161.17, 160.75, 56.01(2), 51.39 (2), 47.91, 46.36, 46.11, 46.08, 36.09, 36.04, 35.99, 35.72, 34.74, 34.27, 29.51, 29.47, 28.70, 28.67, 27.43, 26.32, 25.86, 25.81, 25.28, 25.22, 15.71 (2), 6.81, 6.77.

CoA-desthiobiotin-thiocarbamate

CoASH (10 mM 700 µl in methanol), desthiobiotin sulfone 5 (200 mM, 350 µl in methanol) and LiOH (100 mM, 140 µl in ddH$_2$O) were mixed at rt. After 2 h, the mixture was precipitated by adding diethyl ether and pelleted by centrifugation. The precipitate was dissolved in methanol and then precipitated again with diethyl ether, and this procedure was repeated three times to remove any residual desthiobiotin sulfone. Finally the precipitate was dissolved in ddH$_2$O and checked for its concentration and purity by analytical reversed C-18 HPLC (buffer A: KH$_2$PO$_4$ 50 mM pH 4.5, buffer B: acetonitrile/water=4/1, a gradient elution from 0% B to 60% B for 30min with a flow rate of 1ml/min monitoring UV absorbance at 260 nm, retention time 19.0 min, 96% purity based on peak area)

MALDI HRMS (M+H$^+$) calcd for C$_{36}$H$_{63}$N$_{11}$O$_9$P$_3$S 1078.3230, found m/z 1078.3172.

$^1$H NMR (400MHz, D$_2$O) d 8.37 (s, 1H), 8.06 (s, 1H), 5.97 (d, J=7.2Hz, 1H), 4.65 (m, 1H), 4.60 (m, 1H), 4.39 (m, 1H), 4.04 (m, 2H), 3.83 (s, 1H), 3.66 (m, 2H), 3.54 (m, 1H), 3.35 (dd, J=4.8, 9.6Hz, 1H), 3.26 (t, J=7.6Hz,), 3.17-3.21 (m, 2H), 3.16 (t, J=6.4Hz), 2.97 (m, 2H), 2.75 (m, 5H), 2.25 (t, J=6.4Hz, 2H), 2.03 (t, J=7.2Hz, 2H), 1.61 (m, 1H), 1.53 (m, 1H), 1.39 (m, 2H), 1.26 (m, 2H), 1.11 (m, 4H), 0.88 (d, J=6.4Hz, 2H).
To an ice-bath cooled aqueous solution of the above CoA-desthiobiotin-thiocarbamate (700 µl, 10 mM) was added Oxone® (466 µl, 50 mM aqueous solution). The reaction mixture was incubated at rt for 20 min and after dilution with ddH₂O (2 ml) was injected into preparative-reversed phase HPLC column (buffer A: KH₂PO₄ 50 mM, pH 4.5, buffer B: acetonitrile/ddH₂O=4/1, a gradient elution profile: 0% buffer B at 0 min, 0% buffer B at 5 min, 60% buffer B at 35 min, flow rate 10 ml/min, UV absorbance monitored at 260 nm). The fractions eluted at about 24 min were collected, lyophilized and dissolved in ddH₂O. The KH₂PO₄ salt was precipitated by adding the same volume of acetone and removed by centrifugation. After the acetone was removed under reduced pressure, the aqueous solution of the product was lyophilized and dissolved in ddH₂O to determine its concentration by UV absorbance at 260 nm and its purity by analytical HPLC (the same method used for the starting material; total 6 mL of 865 µM in 92% purity based on HPLC peak area).

MALDI HRMS (M-H+2K⁺) calecd for C₃₆H₆₁N₁₁O₂₀P₃SK₂ 1170.2316, found m/z 1170.2297.

H NMR (400MHz, D₂O) d 8.38 (s, 1H), 8.15 (s, 1H), 5.95 (d, J=5.6Hz, 1H), 4.62 (m, 1H), 4.62 (m, 1H), 4.55 (m, 1H), 3.99 (m, 2H), 3.74 (s, 1H), 3.58 (m, 2H), 3.47 (m, 1H), 3.18-3.35 (m, 7H), 3.06 (m, 2H), 2.94 (m, 2H), 2.74 (s, 3H), 2.23 (t, J=7.4Hz, 2H), 1.96 (t, J=7.4Hz, 2H), 1.57 (m, 2H), 1.32 (m, 2H), 1.20 (m, 2H), 1.05 (m, 4H), 0.081 (d, J=6.4Hz, 3H), 0.64 (s, 3H), 0.52 (s, 3H); ³¹P NMR(160MHz) d 1.06 (s), -9.76 (d, J=21.1 Hz), -10.33 (d, J=21.1Hz).

2.

Protein Labeling

Preparation of HeLa nuclear extract

HeLa (human cervical adenocarcinoma) cells were grown to 80% confluency in DMEM supplemented with fetal bovine serum (10%), penicillin (100 µg/µl) and streptomycin (100 µg/µl). Cells were harvested, Dounce-homogenized in lysis buffer (10 mM KCl, 10 mM HEPES pH 7.9, 0.5 mM DTT, 1.5 mM MgCl₂) and centrifuged 5 min at 10,000 x g, to remove the cytoplasmic fraction. The crude nuclei pellet was resuspended in extract buffer (25% glycerol, 0.5 mM PMSF, 0.5 mM DTT, 1.5 mM MgCl₂, 20 mM HEPES, 0.2 mM EDTA) and shaken gently for 30 min. After centrifugation at 20,000 x g for 5 min, the supernatant was transferred and dialyzed against dialysis buffer (0.5 mM PMSF, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM EDTA, 20% glycerol, 20 mM HEPES pH 7.9).

Procedure for labeling proteins with 3'-³²P FactCoA

Purified proteins (about 5 µM at the final concentration) were treated with 200 µM of Fact-CoA, 1 either in the presence of 2.5 mM (or 5 mM) actCoA or not in assay buffer (50 mM HEPES pH 7.9, 50 mM NaCl, 250 µM DTT) at 30 °C for 2 h. The assay was stopped by addition of 5XSDS-PAGE loading buffer (reducing). Proteins were separated by SDS-PAGE and the gel was dried under vacuum. ³²P-labeled proteins were detected by autoradiography.

Stability of compound 2

Stability of compound 2 to thiol attack was checked under the following conditions.

Compound 2 (100 µM final concentration) was incubated in assay buffer (50 mM HEPES, pH 8.0, 100 mM NaCl, with or without 1 mM DTT). The reaction was monitored using analytical RP-HPLC (UV absorbance at 259 nm, a gradient elution from 10% to 60% buffer B for 30 min, Buffer A: 50 mM KH₂PO₄, pH 4.5, buffer B: acetonitrile/ddH₂O=4/1) by observing the disappearance of compound 2 or the
appearance of CoA in the chromatogram. A half-life of compound 2 is observed to be about 1 h in the presence of 1 mM DTT (and >>1 h in the absence of DTT).

**Procedure for labeling recombinant proteins with CoA-des thiobiotin-thiocarbamate-sulfoxide, 2**

Purified proteins (10 µl of 0.5µg/µl) were treated with 25 µM 2 (865 µM stock in ddH₂O) either with or without 3 mM actCoA in assay buffer (50 mM HEPES pH 8.0, 100 mM NaCl, DTT 0.5 mM) at 30 °C for 1 h. The assay was then quenched with a standard 5XSDS-PAGE loading buffer (reducing). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% BSA in TBS with 0.1 % Tween 20 (TBST) at rt, followed by 1 h incubation with HRP-streptavidin in TBST. After four washes with changes every 15 min in TBST, the biotinylated proteins were visualized by enhanced chemiluminescence.

**Procedures for labeling HeLa nuclear extracts with 2**

Protein mixtures (70 µl of 1.60 µg/µl, either HeLa nuclear extract or HeLa nuclear extract spiked with p300-HAT (1/50 by weight) were treated with 50 µM CoA-des thiobiotin-thiocarbamate sulfoxide, 2 and the rest was done as described in the above section.

**Procedures for probing the labeled lysates by Western blotting after streptavidin-agarose affinity purification.**

The labeled lysates (20 µl of 1.9 µg/µl HeLa nuclear extract was treated with 100 µM 2 either in the presence of 5 mM acetyl CoA or not for 1 hr at 30 °C) were treated with 100% trichloroacetic acid (final concentration 10 %), incubated on ice for 30 min then centrifuged at 14K rpm for 15 min. After removing the supernatant, the pellet was washed with cold acetone and dried at rt in the fume hood for 20 min. The dried pellet was dissolved in TBS-T (0.2 % SDS) heating at 65 °C for 15 min and incubated with streptavidin-agarose for 1 hr at rt. The supernatant was removed by centrifugation at 7K rpm for 5 min and the remaining beads were washed three times with TBS-T (0.1% tween, 10 times volume). Finally, the biotinylated proteins on beads were eluted boiling in 2xsample buffer for 5 min. The eluted proteins were separated on SDS-PAGE gel and transferred to nitrocellulose and the membrane was blocked with 5% nonfat milk in TBST for 1 hr at 5 °C and then incubated with anti-Hat1 antibody in blocking buffer for 1.5 hr. After three washes for 20 min in TBST, the membrane was incubated with anti-goat antibody for 1h at rt, and washed with TBST three more times. The captured Hat1 protein was visualized by chemiluminescence.

**3. Trypsin digestion, purification and detection of biotinylated peptides**

**Trypsinization and streptavidin enrichment**

The labeled proteins (1 µM of p300-HAT, yESAI, were treated with 25 µM CoA-des thiobiotin thiocarbamate sulfoxide 2 as described above) were dialyzed against TBS to remove 2. The dialysate was digested overnight at 37 °C with trypsin, then incubated with streptavidin-agarose beads for 1 h at rt. The beads were then washed 3 times with 10 volumes of low salt wash buffer TBS (50 mM Tris pH 7.4, 150 mM NaCl) and 3 times with high salt wash buffer (50 mM Tris pH 7.4, 500 mM NaCl) and finally with 10 volumes of ddH₂O (twice). After washing, the beads were eluted with 30% aqueous acetonitrile with 0.5% trifluoroacetic acid (TFA). The eluted peptides were partially dried by vacuum centrifugation and analyzed by either MALDI-TOF or LC-MS/MS.
MALDI

Peptide samples were combined with the MALDI matrix (α-cyano-4 hydroxycinnamic acid, 20% acetonitrile, 0.1% TFA in water). Spectra were acquired on a Perseptive Biosystems Voyager-DE Pro in the reflector mode.

ESI-Q-FTMS/MS and Data Analysis

Peptide samples were fractionated in a linear gradient of 30-70% CH₃CN in 0.1% TFA over 1 h at 1 mL/min using a Jupiter C4 reverse-phase column (4.6 × 150 mm). Fractions were lyophilized and then resuspended in 50 µL of electrospray solvent (49% CH₃CN, 50% water, 1% formic acid) for mass spectrometry analysis. The peptides were directly introduced into a custom built quadrupole-FTMS instrument using an Advion Nanomate 100 for automated nanospray. The ions were externally accumulated for a total of 1 s in an accumulation octapole and shuttled to the ICR cell through a quadrupole that can function either as a simple ion guide or as a selective m/z filter.[15] The targeted species were isolated by Stored Waveform Inverse Fourier Transform (SWIFT)[16] and fragmented by Electron Capture Dissociation (ECD).[17] Collected data were analyzed using THRASH[16] producing sets of intact mass and fragment ion peak list, which were uploaded onto the ProSight PTM web server for single protein mode search (prosightptm.scs.uiuc.edu).[19] The mass difference between the observed ion and the theoretical ion indicates modifications on the observed ions.

4. Purification of wild type p300 HAT domain and p300 HAT domain mutants for acetyltransferase assays

The p300 HAT domain encompassing amino acids 1284-1673 (p300HAT(1284-1673)) and the C1621A mutant were purified by coexpression with the histone deacetylase Sir2 as previously described.[9] The C1621A mutant was generated using the Quik Change mutagenesis system (Stratagene). Subsequently, the entire open reading frame was sequenced to ensure that only the desired mutation had been incorporated into the p300 HAT domain expression construct.

5. Histone acetyltransferase (HAT) assay

The kinetic parameters for p300 HAT (aa 1284-1673) and the C1621A mutant were determined using a previously described gel-based assay that measures the incorporation of a ¹⁴C-labeled acetyl group into a peptide substrate.[20] Enzyme activity for both the wild type and mutant proteins was linear with respect to time and enzyme concentration. The concentration of ¹⁴C-acetyl-CoA was fixed at 20 µM when measuring the steady state kinetic parameters for the peptide substrate, i.e. the H4-15 peptide[20] whereas the concentration of the H4-15 peptide was fixed at 150 µM when measuring the steady state kinetic parameters for acetyl-CoA. Assays were performed in duplicate and these generally agreed within 20%. The initial rates obtained from these assays were fitted by non-linear least fit squares to Equation 1,

\[ v = \frac{V_{\text{max}}[S]}{(K_m + [S])} \]  

(Equation 1),

using the KaleidagraphTM version 3.5 software package.
Figure S1. A. In vitro labeling of proteins with 3'-$^{32}$P-Fact-CoA (or 3'-$^{32}$P-Fact-CoA in the presence of actCoA). B. In vitro labeling of cell extracts with 3'-$^{32}$P-Fact-CoA (or 3'-$^{32}$P-Fact-CoA in the presence of actCoA)
Supplementary References


